Induction of apoptosis by all-trans retinoic acid in the human myeloma cell line RPMI 8226 and negative regulation of some of its typical morphological features by dexamethasone

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Abstract

We investigated the effects of all-trans retinoic acid (RA) and dexamethasone (Dex) on the *in vitro* growth of the human myeloma cell line RPMI 8226. RA inhibited RPMI 8226 cell growth by both antiproliferative effect and induction of apoptosis. Typical morphological and biochemical characteristics of apoptosis including chromatin condensation, apoptotic bodies formation and internucleosomal DNA cleavage were detected after 4 days of treatment with 1 μ M RA. *In situ* TUNEL assay demonstrated that DNA cleavage preceded chromatin condensation. The expression of tissue transglutaminase (tTG), an enzyme proposed to play a role in apoptosis was induced with RA, as shown by both enzymatic assay and *in situ* immunofluorescence detection. Dex, when used alone, had no effect on cell growth and apoptosis. When combined to RA, Dex did not interfere with the RA-dependent inhibition of cell proliferation, but unexpectedly inhibited both quantitatively and qualitatively several morphological and biochemical features of the apoptosis induced by RA. Dex did not affect RA-induced DNA breaks formation but impeded the progression of chromatin condensation and the formation of apoptotic bodies. Interestingly, Dex also inhibited the RA-dependent induction of tTG. RU486, a glucocorticoid antagonist, counteracted all Dex effects. Taken together these data demonstrate that key cytoplasmic and nuclear events occurring during apoptosis are differentially regulated by RA and Dex in myeloma cell line RPMI 8226.

Keywords: apoptosis; dexamethasone; retinoic acid; myeloma; tissue transglutaminase

Abbreviations: RA, all-trans retinoic acid; Dex, dexamethasone; tTG, tissue transglutaminase; CAM, camptothecin; MGG, May Grünwald Giemsa; TUNEL, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling; IL-6, interleukin-6; DMSO, dimethylsulfoxide; DABCO, 1,4-diazabicyclo(2,2,2)octane; TCA, trichloroacetic acid

Introduction

Multiple myeloma remains an incurable malignancy. None of the recent advances in chemotherapy resulted in decisive improvement in the prognosis of the disease and new therapeutic approaches are needed.¹

Retinoids are well known regulators of cell proliferation, cell differentiation and embryonal development.^{2,3} Their potential use for cancer treatment received a renewed interest since the successful introduction of RA as therapy for acute promyelocytic leukemia, and significant clinical responses have also been observed in other malignancies.^{4,5} Sidell *et al*⁶ have reported an inhibitory effect of RA on the growth of a human myeloma cell line, AF-10, via downregulation of IL-6 receptors. This observation was further documented by *in vitro* studies on fresh human myelomatous plasma cells.⁷ RA was also reported to inhibit growth of myeloma cells via a mechanism distinct from IL-6 modulation.⁸ Cell accumulation in cancer can result from either increase in proliferation or failure of cells to undergo apoptosis.⁹ RA has been reported to inhibit the proliferation of various cancer cell lines,^{5,10} but also to induce apoptosis in several models, including HL-60 human leukemia cells.^{11–14}

Dex is one of the most effective agents in myeloma therapy;¹⁵ however, there are conflicting data about its effects on myeloma cells growth.^{16,17}

In this study we investigated the effects of RA and Dex on both proliferation and cell death of a human myeloma cell line, RPMI 8226.¹⁸ RA was demonstrated to induce typical apoptosis in these cells. The expression of several morphological and biochemical features of this apoptosis was surprisingly inhibited in the presence of Dex.

Results

Inhibition of cell growth

The effects of RA, Dex and RU486, an antiglucocorticoid, on the growth of RPMI 8226 cells were evaluated (Figure 1). Treatment with 1 μ M RA alone resulted in significant slowing down of cell growth observable from the second day and leading to a progressive growth arrest which was almost

complete after 6 days. RPMI 8226 cells appeared unresponsive to glucocorticoids, since Dex and RU486 treated cells grew as control cells. Dex had no effect on the growth inhibition induced by RA.

Antiproliferative effect

To document the antiproliferative effect of RA, the rate of DNA synthesis was evaluated. As shown in Figure 2, an inhibitory effect of RA was also evident in [^3H]thymidine incorporation of RPMI 8226 cells: RA suppressed [^3H]thymidine uptake by approximately 75% and neither combination of RA and Dex nor RA+Dex+RU486 modified this effect. Dex and RU486, used alone, were here again ineffective. This result was confirmed by determining the growing fraction of the cell population by using Ki-67 antibody. This antibody reacts with an antigen expressed in all stages of the cell cycle with the exception of G0. In control cells, the percentage of Ki-67 positive cells, evaluated after a 3 days culture was 80% (± 7.6) and did not change during the next 2 days. In RA treated cells, the percentage was 36% (± 7.6) after a 3 days treatment and decreased to 17% (± 2.1) after 4 days (not shown). DNA synthesis arrest is an earlier event when compared to the decrease of the number in Ki-67 positive cells, since suppression of [^3H]thymidine uptake was effective after a 48 h treatment while decrease in Ki-67 positive cells was observed after 3 days. It has been already demonstrated that Ki-67 antigen expression can persist 24 h after cell cycle arrest.¹⁹

Induction of apoptosis

To investigate whether RA-induced inhibition of cell growth was also due to cell death, cell morphology was observed after MGG (May-Grünwald Giemsa) and Hoechst 33258 staining. Cells treated with 1 μM RA showed typical

morphological features of apoptosis (Figures 3B and 4B). 20.6% (± 4.5) of RA-treated cells were apoptotic, contrasting with less than 0.9% (± 0.5) for control cells (Figure 5). Dex and RU486 alone did not induce any apoptotic change (results within values of controls). However when used in combination with RA, Dex inhibited the RA-dependent induction of key morphological changes related to apoptosis (Figures 3–5). When compared to RA-treated cells, cells treated with the combination of RA and Dex were characterized by a striking decrease of the number of cells showing typical apoptotic features, and a strong inhibition of apoptotic bodies formation. However, vacuolisation of the cytoplasm, a common feature of apoptotic cells, was observed in cells treated with RA+Dex. Moreover in most cells the nucleus was looking different from control cells as it appeared partially condensed and fragmented (Figures 3C and 4C). Interestingly these effects of Dex could be counteracted by RU486, which restored the classical morphology of apoptosis induced by RA (Figures 3D and 4D), and were therefore probably mediated by the glucocorticoid receptor.

DNA fragmentation

To ascertain that RA induced apoptosis in RPMI 8226 cells, treated cells were submitted to qualitative analysis of DNA fragmentation. We used a new very sensitive and semi-quantitative ligation-mediated PCR assay for the detection of DNA breaks.²⁰ A typical DNA ladder was observed after treatment with RA (Figure 6A, lane 2), or with RA+Dex or RA+Dex+RU 486 (lanes 3 and 4), whereas after treatment with Dex or RU 486 alone a very faint ladder was obtained

Figure 1 Effects of RA, Dex and RU486, an antiglucocorticoid, on RPMI 8226 cells growth. Cell proliferation was evaluated by direct counting (Trypan blue method). Cells were cultured for 2 days with a single dose of each specified compound (RA 1 μM , Dex 0.5 μM and RU486 2 μM) used alone or in combination. After 2 days, the medium was supplemented to 10% FCS and cells were further incubated for 4 days at 37°C. Living cells were counted daily. Mean values ($n=6$) are shown plotted against time. Differences between RA- and not RA-treated cells were highly significant after day 3 ($P=0.0017$ at day 3 and <0.0001 at day 6).

Figure 2 Effects of RA, Dex, and RU486 on the rate of DNA synthesis. RPMI 8226 cells were incubated in the absence (control) or in the presence of the indicated compounds, used at the same concentration as in Figure 3. [^3H]thymidine incorporation was performed at 48 h for 3 h, as described in Materials and Methods. Data are expressed as the mean $\pm$ S.D. ($n=5$) percentages of c.p.m. incorporated in cells treated with inducing agents versus control. Differences between RA- and not RA-treated samples were highly statistically significant ($P<0.0004$).

Figure 3 Morphologic aspect of RPMI 8226 after treatment with RA. Cells were analyzed by May-Grünwald Giemsa staining at day 6 post induction (magnification $\times 1000$). (A) untreated cells, (B) cells treated with RA ($1 \mu\text{M}$) alone, (C) with RA ($1 \mu\text{M}$) plus Dex ($0.5 \mu\text{M}$) or (D) with RA ($1 \mu\text{M}$) plus Dex ($0.5 \mu\text{M}$) and RU486 ($2 \mu\text{M}$). Typical apoptotic cells (☆), apoptotic bodies (arrowheads) and atypical apoptotic cells (★) are indicated. There was no morphological change in cells treated with Dex or RU486 alone (data not shown)

(lanes 5 and 6). The RA-induced DNA fragmentation was also evaluated by a quantitative assay of the low molecular weight DNA extracted from the lysed cells (Figure 6B). $10.1 \pm 2\%$ of fragmented DNA was found in RA-treated cells and only 0.5% in untreated cells. For cells treated with RA+Dex and RA+Dex+RU 486, values were 8 ± 1.8 and $8 \pm 1.6\%$ respectively. Treatment with Dex or RU 486 alone resulted in a very small, but significant, increase in low molecular weight DNA. Taken together these data indicate that RA induces true DNA fragmentation in RPMI 8226 cells. This phenomenon was not inhibited by Dex treatment contrary to the condensation of chromatin and fragmentation of nuclei observed on MGG or Hoechst stained cytospin cell preparations. Finally the TUNEL method provided more details on this puzzling observation by allowing *in situ* labeling of DNA breaks (Figure 7). RA-treated cells showed a high level of DNA fragmentation in the condensed and fragmented chromatin. In cells treated with RA+Dex, a more diffuse labeling was observed in a significant number of cells where no typical chromatin condensation and nuclear fragmentation could be observed by MGG or Hoechst staining. These data demonstrated that Dex did not inhibit the RA-induced DNA breaks formation and the beginning of chromatin condensation, but impeded the progression of this condensation followed by the fragmentation of nuclei and the formation of apoptotic bodies.

Induction of tissue transglutaminase

tTG has been suggested to be implicated in the formation of apoptotic bodies.²¹ Induction of both enzymatic activity and expression of the protein was studied in RA-treated cells. As shown in Figure 8, the basal level of tTG activity in untreated cells was extremely low and treatment with $1 \mu\text{M}$ RA for 48 h resulted in striking induction of this activity. Dex produced a strong inhibition of this induction, an effect prevented by the addition of RU486. Immunofluorescence studies (Figure 9) demonstrated a very low expression of the protein in untreated cells, whereas a very strong expression was found in RA-treated cells, with the strongest reactivity within apoptotic bodies. This effect was inhibited by Dex and as a rule restored by adding RU486. Taken together these results suggested that Dex downregulated tTG induction through ligand activation of the glucocorticoid receptor.

Flow cytometric analysis

In order to delineate more accurately the relative contribution of the cell cycle arrest and cell death induced by RA, flow cytometric analysis was used. RPMI 8226 cells were analyzed for DNA content after propidium iodide staining (Figure 10). The kinetics of the repartition of cells in the various phases of the cell cycle were modified by RA

Figure 4 Hoechst 33258 staining of RPMI 8226 cells nuclei. Untreated cells (A) and cells treated with either RA (B), RA plus Dex (C) or RA plus Dex and RU486 (D) at the same concentrations as in Figure 3 were analyzed at day 6. Apoptotic cells (arrowheads) and apoptotic bodies (☆) are indicated. In panel (C), there are no apoptotic bodies; only one cell displays typical apoptosis and other cells display atypical apoptosis with presence of DNA in the cytoplasm

Figure 5 Quantification of apoptotic cells after treatment with RA and/or Dex and RU486. RPMI8226 cells treated as described in Figure 4 were stained either by Hoechst 33258 (HOECHST) or by May-Grünwald Giemsa (MGG). In each case the percentage of apoptotic cells was determined in duplicate counts of 400 cells. Mean values $\pm$ S.D. from six independent experiments are shown

treatment. The percentage of cells in G0/G1 increased at J2 and J3, with a decrease in cells in the S and G2/M phases, whereas the percentage of cells in sub-G0/G1 increased slightly at J2 and J3 and more strongly between J4 and J6 (Figures 10 and 11A). Dex and RU 486 apparently did not modify the response to RA, and here again were inactive when used alone. These data are in accordance with the [^{3} H]thymidine incorporation assay. RA inhibits the progression of the cells from G1 to S. Cells accumulate first in G0/G1 and then in sub-G0/G1 and die. Interestingly, RA-induced apoptosis has been reported to be preceded by G1 arrest in human MCF-7 breast cancer cells.²² Sub-G0/G1 cells have lost a significant part of their DNA. The percentage of cells with typical or atypical morphological apoptotic features was determined after 6 days of treatment (Figure 11B and C). The total of dying and dead cells is in each case in good agreement with the percentage of sub-G0/G1 cells measured by flow cytometry (Figure 11A). Interestingly, the amount of dying cells was the same in samples treated with RA or RA plus Dex. However, their way to die was different. Treatment with RA resulted in death through typical apoptosis, whereas in the presence of Dex, death was atypical, without nuclear fragmentation or apoptotic bodies.

Discussion

We demonstrated here that RA modulates RPMI 8226 cell growth by both inhibiting cell proliferation and inducing

A

B

Figure 6 DNA fragmentation analysis. (A) Analysis by gel electrophoresis. RA-induced nucleosomal ladders were visualized after LM-PCR assay as described in Materials and Methods. Human myeloma cells were either untreated (lane 1) or treated for 5 days with 1 μ M RA (lane 2), 0.5 μ M Dex (lane 5) or 2 μ M RU486 (lane 6). RA was also used in combination with Dex or Dex plus RU486 (lanes 3 and 4). Lane 7 represents the size marker ($\times 100$ bp). Because of the addition of the adaptors (48 bp total), the bands are slightly larger than those of a conventional ladder. (B) Quantification of fragmented DNA by the diphenylamine assay as described in Materials and Methods. Results are expressed as the percentage of total DNA from 2×10^7 cells

apoptosis. Surprisingly, an inhibition of the induction of some typical features of apoptosis was observed when Dex was combined to RA.

Conflicting data have been reported about the effect of RA on RPMI 8226 cell growth, some authors observing a clear inhibition of cell proliferation^{23–25} whereas others found no effect.²⁶ We have no clear explanation for these discrepancies which are probably due to the use of different RPMI 8226 subclones. It has been recently reported that according to the passage number, cancer cells could

display divergent response to retinoids and steroids.²⁷ The mechanism by which RA inhibits myeloma cell growth is still not clearly understood. In IL-6 dependent myeloma cell lines, and in myeloma cells freshly isolated from patients, RA has been shown to downregulate IL-6 receptor expression, and was identified as a negative modulator of the IL-6 mediated autocrine/paracrine growth.^{6,7,25,26} However RA has also been reported to inhibit the growth of IL-6 independent myeloma cell lines.⁸ In fact the mechanisms by which retinoids negatively regulate tumor cell growth are largely unknown. A better understanding of these mechanisms will require the characterization of the subtype of receptor involved, RAR and/or RXR,²⁸ and the identification of the target genes involved. Preliminary results from our laboratory suggest that the activation of both RAR and RXR is required to obtain a full response on cell proliferation and apoptosis.²⁹

We did not observe any effect of Dex alone on RPMI 8226 cell growth. Our data are in agreement with previous papers reporting that RPMI 8226 cell growth was insensitive to glucocorticoids.^{16,26} This resistance was attributed to a post-receptor mechanism, since the presence of a functional glucocorticoid receptor was documented in these cells.¹⁶ This result is in accordance with our findings that Dex, despite its lack of effect on RPMI 8226 cell growth, was still able to modulate RA-dependent induction of apoptosis and tTG in these cells. These responses were likely mediated by the glucocorticoid receptor, as suggested by the inhibitory effect of RU486, an antiglucocorticoid acting at the receptor level. Very recently RPMI 8226 cells have been reported to be sensitive to Dex, which inhibited cell growth at low concentrations and induced apoptosis at high (micromolar) concentrations.^{30,31} In a congress abstract, a synergy between RA and Dex has been reported,⁸ in sharp contrast with other reports describing RPMI 8226 as resistant to both agents.²⁶ This discrepancy with previous results could also be due to the use of different RPMI 8226 subclones. If glucocorticoids are among the most effective agents in treating myeloma and inhibit the proliferation of myeloma cells freshly purified from bone marrow aspirates,¹⁷ their effects on myeloma cell lines in culture are far more variable.^{16,30–33} In fact myeloma cells become progressively resistant to glucocorticoids either by acquiring a glucocorticoid receptor mutation³⁴ or more commonly by a post-receptor mechanism.¹⁶ The latter mechanism could affect the expression of only a subset of the different genes normally controlled by glucocorticoids in the fully sensitive cell lines and explain why some other cell lines exhibit dissociated or incomplete responses to glucocorticoid treatment.

Our results demonstrated clearly that RA is able to induce apoptosis in RPMI 8226 cells. Typical morphological and biochemical characteristics of apoptosis³⁵ were observed. When compared to the inhibition of cell proliferation the kinetic of induction of apoptosis appeared slower. Typical apoptotic cells were detected by MGG and Hoechst staining after 4 days of RA treatment. However with the more sensitive TUNEL assay DNA cleavage was already observable *in situ* after 3 days. DNA cleavage has already been demonstrated to occur before chromatin

Figure 7 *In situ* labeling of DNA strand breaks. Untreated cells (A) and cells treated with either RA (B), RA plus Dex (C) or RA plus Dex and RU486 (D) at the same concentrations as in Figure 4 were analyzed at day 6 using the TUNEL assay as described in Material and Methods. Apoptotic cells (★) and apoptotic bodies (arrows) are indicated

A)

B)

SAMPLES (n=5)	%RA	SD
CONTROL	0,6	1,2
RA	100	0
RA+Dex	31,8	8,2
RA+Dex+RU486	82,2	8,6
Dex	1	1,4
RU486	1,2	1,5

Figure 8 Tissue transglutaminase activity in RPMI 8226 cells treated with RA, Dex or RU486. Specific tTG activity (1 U=1 pm of [³H]putrescin incorporated in NN' methylcasein/min/mg of cell proteins at 30°C) was analyzed in cells treated for 48 h with the same concentrations and combinations of agents as in the preceding figures. (A) results of a representative experiment, (B) mean of five different experiments. Results are expressed as a percentage of tTG activity detected after treatment with 1 μM RA. Differences between RA- and RA+Dex-treated samples were highly significant ($P=0.0006$)

condensation in other models.³⁶ RPMI 8226 cells treated with both RA and Dex failed to show any typical chromatin condensation, whereas DNA breaks were detected by both TUNEL assay and DNA electrophoresis, suggesting that these two phenomenon were independently regulated in

our cells. It has been reported that morphological changes typical of apoptosis, like chromatin condensation, could be dissociated from internucleosomal cleavage³⁷ and the role played by DNA fragmentation in the apoptotic process is presently controversial.³⁸ Chromatin condensation during

Figure 9 Indirect immunofluorescence detection of tissue transglutaminase in RPMI 8226. Untreated cells (A) and cells treated with either 1 μ M RA (B) or 1 μ M RA plus 0.5 μ M Dex (C) or 1 μ M RA plus 0.5 μ M Dex and 2 μ M RU486 (D) were analyzed after 6 days of culture (magnification $\times$ 1000). Cells treated with Dex or RU486 alone gave results similar to untreated cells (data not shown). Apoptotic cells (☆) and apoptotic bodies (arrows) are indicated

Figure 10 Kinetics of the effects of RA and Dex on the cell cycle parameters. RPMI 8226 cells were cultured for 6 days after treatment with 1 μ M RA, 0.5 μ M Dex or 2 μ M RU486 used alone or in combination. Flow cytometry DNA histograms after propidium iodide staining were recorded and analyzed. The percentage of cells in the various phases of the cell cycle, i.e. G0/G1, S and G2/M, as well as of polyploid cells and sub-G1 cells was determined in all samples after various culture times (2–6 days)

apoptosis is accompanied by degradation of lamin A+B.³⁹ We started an immunocytochemical study of the expression of lamin B during apoptosis in our cells. Whereas treatment with RA alone resulted in extensive lamin B degradation concomitant to chromatin condensation, in the presence of RA plus Dex lamin B was only partly dissociated (data not shown).

In RPMI 8226 myeloma cells, the basal level of expression of tTG is negligible and RA treatment induces expression detectable after a few hours at the mRNA level (data not shown) and after 2 days at both the protein and enzymatic activity levels (Figures 8 and 9). The control of expression of tTG by RA is well documented in other human and animal models,⁴⁰ where it appears to be transcriptional.⁴¹ Conflicting data have been reported about the type of RA receptors involved in this control.^{14,42} Whereas a retinoid response element has been identified within the mouse tTG gene promoter,⁴³ such a responsive element has not yet been found in the human promoter sequence.⁴⁴ Moreover no negative glucocorticoid element has been identified in this promoter to date, and the mechanism of the inhibition of the RA-dependent induction of tTG is still unknown. tTG is a Ca^{2+} -dependent enzyme catalyzing the formation of ϵ -(γ -glutamyl) lysine cross-links between polypeptide chains⁴⁵ and could play a role in apoptosis by cross-linking cellular proteins and preventing the release of intracellular macromolecules.²¹ Our observation that this enzyme accumulates in apoptotic bodies is in agreement with previous observations suggesting that this enzyme could participate in their formation.²¹ Overexpression of tTG in human neuroblastoma cells renders these cells highly susceptible to apoptosis suggesting a direct effect of tTG in the phenotypic maturation process leading to apoptosis.⁴⁶ Interestingly, in our experiments, Dex inhibited the RA-dependent induction of tTG expression and of some of the morphological hallmarks of apoptosis (namely the apoptotic bodies), reinforcing the hypothesis that a functional link exists between the two phenomena. The association of a lack of tTG activity and of a poorly organized apoptotic morphology in Fas-induced apoptosis in thymocytes is in agreement with this hypothesis.⁴⁷ The ability of tTG to play an effector role in the apoptotic program depends on the cellular context.⁴⁸ In our RPMI 8226 cells as in HL-60 leukemia cells, both tTG expression and apoptosis are induced following RA treatment,^{13,42} whereas in other cellular models, like NB-4 human acute promyelocytic leukemia cells, RA-dependent tTG induction may be observed without any occurrence of apoptosis.⁴⁹ tTG regulation in apoptosis probably requires cooperation with other effector agents, which may be differentially expressed and controlled according to the cell type studied.

A growing attention is focused on apoptosis in multiple myeloma. It has been recently shown that apoptosis could be elicited in certain myeloma cell lines by various culture conditions, including serum starvation and treatment with antimitotic agents or with glucocorticoids, an effect prevented by interleukin-6, a major myeloma growth factor.^{30–32} RA was also recently reported to interfere, in long-term culture experiments, with the IL-6 autocrine

Figure 11 Kinetics of the appearance of sub-G0/G1 cells and ratio of apoptotic to necrotic cells after treatment with RA and/or Dex. Cells were cultured for 6 days with, at J0, a single pulse of each inducer (1 μ M RA, 0.5 μ M Dex, 2 μ M RU486) used alone or in combination. (A) Cells (percentage of total) in the sub-G0/G1 fraction measured from J2 to J6 by flow cytometry after propidium iodide staining. Quantification of typical and atypical apoptotic cells at J6 was performed after staining with May-Grünwald-Giemsa (B) and by using the TUNEL method (C). In each case, percentages were determined in triplicate counts of 400 cells

growth of the two human myeloma cell lines U266 and RPMI 8226, via induction of apoptosis.²⁵ Beyond this cross-talk with the IL-6 pathway we are trying now to define how RA can interact with the expression of various elements of the complex network of cell death regulators.^{50,51}

Finally, RPMI 8226 cells afford a good model to study the differential regulation of the various cytoplasmic and nuclear events occurring during apoptosis. Other cell lines should be explored in order to assess the potential relevance of the effects observed in this peculiar model to the regulation of myeloma death, in general, and the implications for myeloma therapy.

Materials and Methods

Reagents

RA, Dex (both from Sigma, St Louis, MO, USA) and RU486 (Roussel Uclaf, Romainville, France) were dissolved in absolute ethanol at 10^{-2} M stock concentration and stored at -20°C in the dark. The final ethanol concentration in culture medium was below 0.01%. Camptothecin (Sigma) was dissolved in dimethylsulfoxide (DMSO) at a initial stock concentration of 3×10^{-3} M and stored at -20°C in the dark.

Cell lines and cell culture

RPMI 8226 cells (human myeloma, CCL-155 American Type Culture Collection, USA)^{18,52} were maintained as suspension cultures in RPMI media, supplemented with Glutamax I, 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μ g/ml streptomycin (all from Gibco-BRL, Paisley, Scotland). Cells were maintained in logarithmic growth at a cell density of about 5×10^5 cells/ml. Cells mean doubling time was around 48 h.

Cells in logarithmic phase were resuspended in culture medium (RPMI 1640 containing Glutamax I, 100 U/ml penicillin, 100 μ g/ml streptomycin, 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml sodium selenite (Sigma) and supplemented with 5% FCS) at a density of 3×10^5 cells/ml into culture flasks protected from light. Cells were treated with 10^{-6} M RA, 0.5×10^{-5} M Dex, 2×10^{-6} M RU486, alone or combined, or with vehicle only. After 48 h of contact, the medium was adjusted to 10% FCS and cells were diluted daily to maintain a concentration of 5×10^5 cells/ml for 6 days.

Proliferation assays

[³H]thymidine incorporation assay After 48 h treatment, 0.25 ml cell samples (2×10^5 cells/ml) resuspended in complete medium were distributed in 24-well plates. [³H]thymidine (20 Ci/mmol, Amersham, UK) was then added at 1 μ Ci/well for 3 h. Cells were washed twice with ice cold Earle's medium (Gibco-BRL) and incubated overnight at 4°C with 50 μ l of lysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA). 1 N NaOH was added to the lysate. Incubation proceeded for 1 h at 4°C then the lysate was neutralized by 50 μ l HCl. Proteins and DNA were precipitated by 5% TCA (4°C , 30 min) and collected over a GF/C (Whatman, Paris, France) glass fiber filter. Thymidine incorporation was assayed by scintillation counting of the GF/C filters (average thymidine incorporation in untreated cells was usually 8600 c.p.m./well).

Ki-67 detection by indirect immunocytochemistry Percentage of Ki-67 positive cells was evaluated after 2–5 days of treatment. Cells spread on glass slides were washed three times with $1 \times$ PBS, fixed by cold-acetone (buffered at pH 7.6) and by absolute cold-acetone (each for 2 min at -20°C). After drying for 10 min at room temperature, cells were fixed again with 4% paraformaldehyde (Merck) in $1 \times$ PBS for 2 min, permeabilized in 0.1% Triton X-100 in $1 \times$ PBS for 5 min and then incubated with reagents diluted in $1 \times$ PBS-3% BSA. Cells were labeled with successive layers of mouse monoclonal IgG anti-Ki-67 (MIB 1, Immunotech, Marseille, France) and avidin/biotinylated enzyme complex (Vectastain Elite-ABC Peroxidase kit, Vector laboratories, INC, USA). DAB (Sigma) was used as the peroxidase substrate. Cells were stained with diluted Giemsa.

Morphological studies

Cell viability was determined using the trypan blue exclusion test. Cells were counted with a Malassez hemocytometer over the 6 days treatment (living and apoptotic cells excluded trypan blue).

After the appropriate time of treatment, 10^5 cells were centrifuged on glass slides (Cytospin 3, Shandon, Cergy Pontoise, France), air-dried for at least 2 h and stored at -20°C . Cells were stained with MGG. DNA staining with Hoechst 33258 [Bis (benzidine)] (Sigma) was performed as described by Galli and Fratelli.⁵³ Briefly, cells were fixed by Carnoy solution (methanol:acetic acid, 3:1, v/v), stained with Hoechst 33258 (0.1 μ g/ml in $1 \times$ PBS for 10 min at 37°C), washed with tap water for 5 min, air-dried and mounted with DABCO-glycerol (1,4-diazabicyclo(2,2,2)octane, Aldrich, Milwaukee, WI, USA). Cells were observed using an Olympus BH fluorescence microscope

(excitation wavelength: 365 nm). The number of apoptotic cells was determined after counting at least 2000 cells.

Apoptotic cells were identified upon the following morphological features:³⁵ cells displaying chromatin condensation, nuclear disintegration, vacuole formation and membrane blebbing were identified as apoptotic. By contrast, cells with cytoplasmic swelling, membrane disruption and disappearance of nuclear chromatin or distribution of the nuclear chromatin in small ill-defined particles, could be early classified as lysed.

DNA fragmentation analysis

Total genomic DNA was isolated from 2.5×10^6 cells using the method described by Eldadah *et al.*⁵⁴ Briefly, the cells treated or not were collected by centrifugation at day 5, washed once with PBS and lysed by 2 ml of 7 M guanidinium hydrochloride. The lysate was added to 1 ml of Wizard Minipreps DNA Purification Resin (Promega), gently mixed and centrifuged at $2000 \times g$ for 5 min. The pellets were resuspended in 3 ml of the following solution: 90 mM NaCl, 9 mM Tris-HCl pH 7.4, 2.25 mM EDTA, 55% ethanol, and transferred to Wizard Minicolumns (Promega). The columns were washed with 2×3 ml of the same solution and centrifuged over microfuge tubes at $5000 \times g$ for 5 min. DNA was eluted by 50 μ l TE buffer pH 8.0 and residual RNA was eliminated by incubation with RNase A at 37°C for 30 min.

The DNA obtained was of high purity, with a 260:280 nm ratio of 1.8–2.0. It was used in a LM-PCR assay (Ligation Mediated Polymerase Chain Reaction),²⁰ according to the manufacturer's instructions (Clontech Laboratories, Palo Alto, CA, USA). This method allows to reveal genomic DNA ladders that may be undetectable by other methods. DNA electrophoresis (10 ng/lane) was run through 1.2% agarose/EtBr gel at 6 V/cm for approximately 2.5 h. Because of the addition of the LM-PCR adaptors (48 bp total) the bands are slightly larger than those of a conventional ladder.

Quantification of DNA fragmentation

Solubilized DNA was quantified by the diphenylamine assay as described by Bansal *et al.*⁵⁵ Treated and untreated cells (2×10^7) were harvested, washed at 4°C in $1 \times$ PBS and lysed for 30 min at 4°C in 5 mM Tris/HCl pH 8.0, 20 mM EDTA buffer containing 0.5% Triton X-100. Lysates were centrifuged at $27\,000 \times g$ for 20 min to separate low molecular weight DNA (supernatant) from intact chromatin (pellet). Pellet and supernatants were resuspended in 500 μ l of lysis buffer supplemented with 2 mg/ml BSA. After adding 1 ml 10% trichloroacetic acid (TCA), and incubation for 20 min at 4°C, samples were centrifuged for 5 min at $10\,000 \times g$. Pellets were resuspended in 1 ml 100% ethanol at 4°C and incubated overnight at -20°C . After centrifugation at $18\,000 \times g$ for 30 min, RNA was hydrolysed in 1 N NaOH at 37°C for 1 h. Reaction mixtures were incubated for 15 min on ice, brought to 10% TCA (30 min at 4°C) and centrifuged at 4°C for 10 min at $8000 \times g$. Pellets were resuspended in 1 ml of 5% perchloric acid for 15 min at 90°C and frequently mixed. Samples were centrifuged for 20 min at $900 \times g$ at 4°C and supernatants were treated overnight at 37°C with 2 ml Burton's reagent.⁵⁶ OD₆₀₀ of each sample was assayed and the DNA amount found in supernatants was expressed as the percentage of total DNA from 2×10^7 cells.

TUNEL technique

In situ nuclear DNA fragmentation was measured according to a method based on 3'OH end labeling of DNA breaks with deoxyuridine terminal deoxynucleotidyl transferase (TdT),⁵⁷ with minor modifications. Treated or untreated cells were spread on glass slides, rehydrated

with $1 \times$ PBS for 5 min, fixed with 1% paraformaldehyde (pH 7.4) (Merck, Darmstadt, Germany) for 15 min at 4°C, and washed twice with $1 \times$ PBS. The TUNEL reaction was carried out by incubating cells for 1 h at 37°C in a moist chamber with 1 nmol/ μ l FITC-12-dUTP (Boehringer Mannheim), 25 mM CoCl₂, 25 U/ μ l TdT (Boehringer Mannheim) and TdT buffer (125 mM Tris-HCl, 1 M potassium cacodylate, 1.25 mg/ml BSA, pH 6.6). Cells were then washed twice with ice-cold $1 \times$ PBS, stained with Evans blue and mounted with DABCO-glycerol. Fluorescence was observed using the fluorescence microscope.

Tissue transglutaminase

Immunofluorescence staining Treated or untreated cells were processed on slides as described by Collard *et al.*⁵⁸ and incubated with the primary antibody, a specific mouse monoclonal anti-tTG IgG raised against purified guinea pig enzyme⁵⁹ (generous gift from Dr P. Birckbichler, Ardmore, Oklahoma, USA). The secondary antibody was fluorescein-conjugated sheep anti-mouse IgG, used at a dilution of 1/1000 (Biosys, Compiègne, France). Slides were observed using the fluorescence microscope.

Activity assay tTG activity of cell lysates was assayed by Ca²⁺-dependent incorporation of [³H]putrescine into N,N' dimethylated casein.⁶⁰ 1×10^7 treated or untreated cells were harvested, washed twice with $1 \times$ PBS and lysed in ice cold lysis buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.5% Triton X-100 for 30 min. 0.2 mg protein were incubated at 30°C for 20 min in a total volume of 100 μ l containing 20 mM Tris-HCl pH 7.5, 5 mM CaCl₂, 10 mM b-mercaptoethanol, 2 mg/ml N,N' dimethylated casein, 2 μ Ci [³H]putrescine (30–60 Ci/mmol, Dupont-NEN), 0.2 mM putrescine. Aliquots were spotted on Whatman 3MM filter paper, fixed in 10% TCA and then sequentially washed in 5% TCA, ethanol/acetone (v/v) and acetone. Protein-bound [³H]putrescine was determined by liquid scintillation counting of the filters. Background value was obtained by substitution of 1 mM EGTA for CaCl₂ in the reaction mixture. Enzyme activity was expressed in picomoles of [³H]putrescine incorporated into N,N' dimethylated casein per minute and per milligram of protein. Protein concentration was assayed by the Bradford method⁶¹ using fraction V Albumin (Boehringer Mannheim) as a standard.

Cell cycle analysis and flow cytometric detection of apoptosis

10^6 cells were collected in triplicate by centrifugation from treated and control cultures, washed once with PBS, fixed in 70% cold ethanol and stored at -20°C . On the day of analysis, after washing with PBS, cells were treated with 5 U/ml RNase A (DNase-free, Promega) for 15 min at room temperature. They were then incubated for 30 min in the dark with 50 μ g/ml propidium iodide (Sigma Chemical, St Louis, MO, USA) containing 0.01% Triton X-100. DNA content was analyzed immediately using a Coulter (Hialeah, FL, USA) EPICS ELITE flow cytometer. Upon acquisition of 20 000 events, the percentage of cells in the different phases of the cell cycle was determined, using the Multicycle analysis software (Coulter). Apoptosis was also quantified: the hypodiploid region ($<2n$) was identified as apoptotic cells and appeared as a distinct sub-G0/G1 peak.

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